

Diffusivity Measurements of Human Methemoglobin

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ABSTRACT Experimental measurements of the diffusion coefficient of human methemoglobin were made at 25°C with a modified Stokes diaphragm diffusion cell. A Millipore filter was used in place of the ordinary fritted disc to facilitate rapid achievement of steady state in the diaphragm. Methemoglobin concentrations varied from approximately 5 g/100 ml to 30 g/100 ml. The diffusion coefficient in this range decreased from 7.5×10^{-7} cm²/sec to 1.6×10^{-7} cm²/sec.

INTRODUCTION

To understand the mechanism of facilitated oxygen diffusion through hemoglobin solutions, it is necessary to know the diffusion coefficient of hemoglobin itself (1, 5, 12). For human hemoglobin, the only measurements of this quantity reported in the literature are those of Lamm and Polson (8) which are limited to dilute hemoglobin solutions. Estimates of the diffusivity at higher concentrations (7, 11) indicate a marked decrease of diffusivity with concentration emphasizing the need for more experimental measurements.

This paper reports the results of a study (4) made to gather such data over a broad range of hemoglobin concentrations. The data were subsequently used to establish the mechanism of facilitation in the transport of oxygen through hemoglobin solution (5). Measurements were made in a diaphragm diffusion cell with human methemoglobin solutions. It has been suggested (12) that an apparent flux of oxyhemoglobin can result from the transfer of oxygen molecules between adjacent hemoglobin molecules in much the same way as an apparent flux of hydronium ions results from the transfer of protons between adjacent water molecules (10). By using methemoglobin, this artifact was avoided. Since the architecture of the methemoglobin molecule is similar to that of the oxyhemoglobin molecule and only slightly different from that of the reduced hemoglobin molecule (9), the diffusivities of all three forms should be nearly equal.

EXPERIMENTAL EQUIPMENT AND PROCEDURES

The principles of the diaphragm cell method are discussed at length by Gordon (2). Fundamentally, the apparatus consists of two well stirred reservoirs separated by a porous diaphragm. The concentration of the diffusing substance differs in the two chambers. After calibrating the diaphragm, the diffusion coefficient is determined by measuring concentration change with time in the reservoirs. A diagrammatic representation of the diaphragm cell is shown in Fig. 1. It was constructed of Lucite and the volume of each chamber was approximately 15 ml. For the porous diaphragm

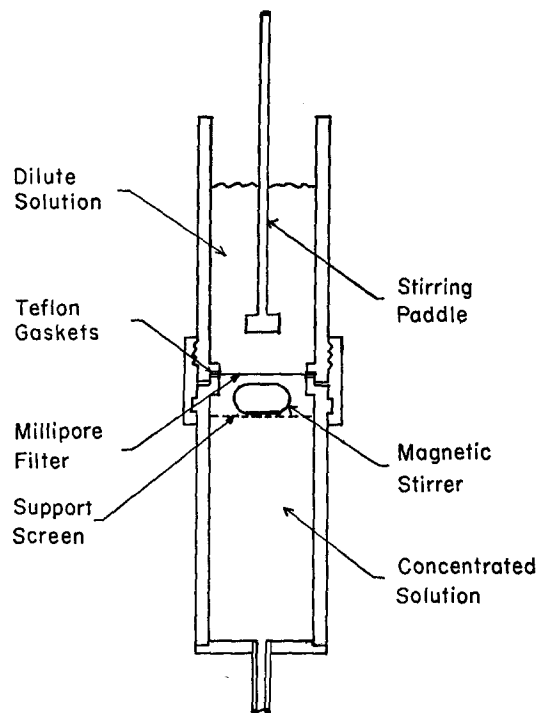


FIGURE 1. Modified Stokes diaphragm diffusion cell for methemoglobin diffusivity measurements.

a Type RA Millipore filter was used. These filters are 150 microns thick, have a porosity of 82%, and a pore diameter of 1.2 microns. The diameter of the exposed portion of the filter was 1.6 cm.

The cell was oriented vertically with the hemoglobin solution in the bottom and phosphate buffer in the top chamber. By placing the denser solution in the lower reservoir, transfer across the diaphragm by bulk mixing was minimized. Leakage around the edges of the Millipore filter was prevented by placing 0.01 in. Teflon gaskets above and below the filter. The contents of the bottom chamber were stirred with a magnetic stirrer supported on a coarse stainless steel screen just below the filter. The stirrer was rotated at 65 RPM. In the upper chamber, mixing was accomplished by a mechanical stirrer, inserted from the top, rotating at approximately 100 RPM. This arrangement prevented physical contact between the stirrers and the rather fragile Millipore filter.

The thickness of the Millipore filter was about $\frac{1}{10}$ the thickness of the fritted glass disc ordinarily used in diaphragm diffusion cells. Since the length of time to steady state is proportional to the square of the thickness, this thinner diaphragm decreased the transient time by a factor of 100. At the relatively low values of the diffusion coefficient expected for hemoglobin solutions, this meant that the transient part of the run would last less than an hour rather than several days. Since the hemoglobin solutions could not be kept indefinitely at room temperature without denaturation, this was especially significant.

The cell was initially filled from the bottom, allowing the test solution to rise through the Millipore filter. This avoided trapping air in the filter. The solution was then removed by pipette from the top chamber, the chamber rinsed, and refilled with 10 ml of 0.05 M, pH 7.4 phosphate buffer. The cell was placed in a $25^\circ \pm 0.02^\circ\text{C}$ water bath and the stirrers started. After approximately 45 min the solution in the top chamber was removed and replaced with 10 ml of fresh buffer. In this first period a steady-state distribution was established in the Millipore filter. The steady-state experimental measurements were then made by periodically removing and replacing the solutions in the upper chamber. The solutions removed were analyzed with a Bausch and Lomb Spectronic 20 colorimeter to determine the methemoglobin concentration.

The relation between the flux of methemoglobin through the diaphragm and the diffusivity is determined by considering the porous diaphragm as a one-dimensional, steady-state system with quasi-steady-state boundary conditions. Gordon (2) has given a solution for the case in which the driving force across the membrane changes significantly from the beginning to the end of the run. Because of the high absorption coefficient of methemoglobin, very small concentration changes in the upper reservoir could be detected and the concentration gradient change during the experiments was limited to less than 1 %. Thus, the driving force could be assumed constant during the run and, with an error of less than 1 %, the following relation for the integral diffusion coefficient could be used:

$$\bar{D}_i \left(\frac{A}{\delta} \right) (n_{i2} - n_{i1})t = v_1 n_{i1} \quad (1)$$

Here v_1 is the volume of the upper reservoir, A/δ is the area to length ratio of the porous diaphragm, and n_{i1} and n_{i2} are the final concentrations in the upper and lower reservoirs respectively. Since n_{i1} is negligible compared to n_{i2} , Equation (1) can be approximated as follows:—

$$\bar{D}_i \left(\frac{A}{\delta} \right) = \frac{v_1 n_{i1}}{n_{i2} t} \quad (2)$$

Gordon has discussed errors in the quasi-steady-state approach related to the volume of the membrane and the homogeneity of the solutions in the reservoirs; these errors were negligible in the present experiments. The error due to diffusion-induced convective transport is discussed by Gosting (3). In the case of methemoglobin and water, the error resulting from this effect is less than the experimental error.

Each time the hemoglobin solution in the lower chamber was replaced, the Millipore filter was also changed. This was necessary since the filter was handled while emptying the cell and its characteristics were altered significantly. This change of filter introduced a small error due to variations in the A/δ ratio from filter to filter. However, the reproducibility of results indicated that this variation was not significant.

Hemoglobin solutions were prepared from human blood which had been in storage in the blood bank of The Johns Hopkins Hospital for 3 wk prior to use. The procedure for preparing solutions of oxyhemoglobin is described elsewhere (5). The oxyhemoglobin solutions were converted to methemoglobin by adding 0.03 g of potassium ferricyanide per gram of oxyhemoglobin. This is 50 % in excess of the stoichiometric requirement; additional potassium ferricyanide caused no additional oxidation of the

TABLE I
INTEGRAL DIFFUSION COEFFICIENT
OF METHEMOGLOBIN AT 25°C

MHb concentration	$\overline{D}_{\text{MHb}}(A/\delta)$
<i>g/100 ml</i>	<i>cm²/sec × 10⁵</i>
2.41	3.92
4.64	3.90
14.3	3.04
14.2	3.26
18.7	2.67
18.8	2.73
21.1	2.53
21.1	2.48
27.4	2.27
27.4	2.13

hemoglobin, confirming that oxidation was complete. The excess ferricyanide was left in the hemoglobin solutions. Concentrations were determined by measuring the per cent transmittance at 500 m μ . The colorimeter was calibrated by the pyridine hemochromagen method (5). Blank runs with potassium ferricyanide solutions indicated that the concentrations of ferricyanide in the sample would not interfere with measurements. Solution concentrations were checked before and after each run to insure that no changes in the methemoglobin had occurred.

EXPERIMENTAL RESULTS AND DISCUSSION

The experimental data are shown in Table I and plotted in Fig. 2 as $\overline{D}_{\text{MHb}}(A/\delta)$ vs. methemoglobin concentration. In dilute solution, the value of $\overline{D}_{\text{MHb}}$ should approach 7.5×10^{-7} cm²/sec, an extrapolation to 25°C of the value measured by Lamm and Polson (8). This value corresponds to a Stokes-Einstein radius of 29 Å. From the intercept of the curve in Fig. 2, and Lamm and Polson's measured value for $\overline{D}_{\text{MHb}}$, the value of A/δ was determined to be 53.3 cm. The reproducibility of the results for different filters at the same methemoglobin concentration indicates that the value of A/δ varied little from filter to filter.

Based on the nominal porosity and thickness and exposed diameter of the Millipore filter, the calculated value of A/δ is 87 cm. To make this calculation one assumes that the pores are of constant cross-section, straight through, and have axes normal to the surface of the filter; since this is probably not

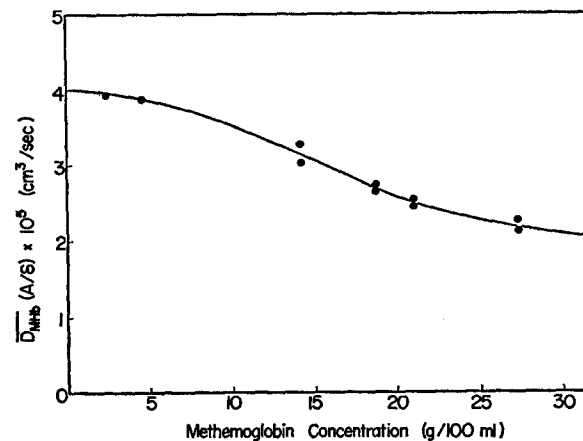


FIGURE 2. Integral diffusion coefficient of methemoglobin at 25°C.

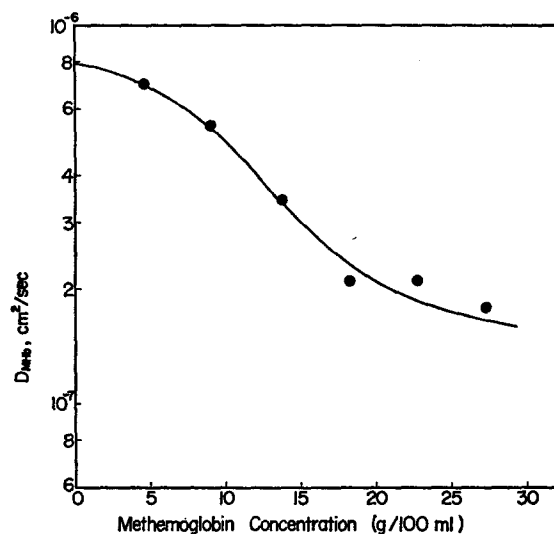


FIGURE 3. Differential diffusion coefficient of methemoglobin at 25°C calculated from integral diffusion coefficient measurements.

so, the actual value would be expected to be somewhat lower, as the experimental data indicate.

From Fig. 2 and the value of A/δ obtained by calibration, it is possible to calculate values of the differential diffusion coefficient. The integral dif-

fusion coefficient, as defined by Equation (1), is related to the differential diffusion coefficient, D_i , in the following manner (2):

$$\overline{D}_i(n_{i2} - n_{i1}) = \int_{n_{i1}}^{n_{i2}} D_i dn_i \quad (3)$$

During the experimental runs, the value of n_{MHB_1} never exceeded 1% of n_{MHB_2} . Under the circumstances Equation (3) can be adequately approximated as follows:—

$$\overline{D}_{\text{MHB}} n_{\text{MHB}_2} = \int_0^{n_{\text{MHB}_2}} D_{\text{MHB}} dn_{\text{MHB}} \quad (4)$$

Differentiating Equation (4), one obtains

$$D_{\text{MHB}_2} = \overline{D}_{\text{MHB}} + n_{\text{MHB}_2} \frac{d\overline{D}_{\text{MHB}}}{dn_{\text{MHB}_2}} \quad (5)$$

The slope $d\overline{D}_{\text{MHB}}/dn_{\text{MHB}}$ was obtained from the curve of Fig. 2. Values of the differential diffusion coefficient were then calculated and plotted in Fig. 3. In view of the uncertainty in fitting the curve of Fig. 2 to the data, the values of the slope may be in error. The relative magnitudes of the terms in Equation (5) are such that an error of 10% (maximum expected) in determining the slope of Fig. 2 would have a negligible effect on the value of D_{MHB} at concentrations less than 10 g/100 ml. A maximum error of about 15% would be expected at 30 g/100 ml.

Roughton has estimated that the diffusion coefficient of hemoglobin in a 35 g/100 ml solution at 25°C is 2×10^{-8} cm²/sec (11). A smooth extrapolation of the curve of Fig. 3 would indicate a value of approximately 1.5×10^{-7} cm²/sec. However, a comparison between these two values is not necessarily valid since the experimental data upon which Roughton's estimate is based (Klug and coworkers, 6) indicate a rapid drop in diffusivity starting at about 25 g/100 ml. Thus the smooth extrapolation to values above 25 g/100 ml is questionable. Roughton has not attempted to estimate the hemoglobin diffusion coefficient at lower concentrations where a comparison with these experiments would be possible.

NOMENCLATURE

A	cross-sectional area for flow through porous diaphragm, cm ² .	D_i	differential diffusion coefficient of the i^{th} species, cm ² /sec.
		\overline{D}_i	integral diffusion coefficient of the i^{th} species, cm ² /sec.

n_i	concentration of the i^{th} species, g-moles/cm ³ .		<i>Subscripts</i>
t	time, sec.	1	value in the upper or dilute reservoir.
v_1, v_2	volumes of upper and lower reservoirs respectively, cm ³ .	2	value in the lower or concen- trated reservoir.
δ	diffusion path length, cm.		

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REFERENCES

1. FATT, I., and LAFORCE, R. C., *Science*, 1961, **133**, 1919.
2. GORDON, A. R., *Ann. New York Acad. Sc.*, 1945, **46**, 285.
3. GOSTING, L. J., *Adv. Protein Chem.*, 1956, **11**, 438.
4. KELLER, K. H., Ph.D. Dissertation, The Johns Hopkins University, Baltimore, 1964.
5. KELLER, K. H., and FRIEDLANDER, S. K., *J. Gen. Physiol.*, 1966, **49**, 663.
6. KLUG, A., KREUZER, F., and ROUGHTON, F. J. W., *Helv. Physiol. et Pharmacol. Acta*, 1956, **14**, 121.
7. LAFORCE, R. C., and FATT, I., *Tr. Faraday Soc.*, 1962, **58**, 1451.
8. LAMM, O., and POLSON, A., *Biochem. J.*, 1936, **30**, 528.
9. PERUTZ, M. F., in *Hemoglobin*, (F. J. W. Roughton and J. C. Kendrew, editors), London, Butterworth & Co., Ltd., 1949, 135.
10. ROBINSON, R. A., and STOKES, R. H., *Electrolyte Solutions*, London, Butterworth & Co., Ltd., 2nd edition, 1959, 120.
11. ROUGHTON, F. J. W., *Progr. Biophysics and Biophysic. Chem.*, 1959, **9**, 55.
12. SCHOLANDER, P. F., *Science*, 1960, **131**, 585.